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The effect of *Rickia wasmannii* (Ascomycota, Laboulbeniales) on the aggression and boldness of *Myrmica scabrinodis* (Hymenoptera, Formicidae)

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Abstract

The interactions of ectosymbiotic Laboulbeniales (Ascomycota) fungi and their hosts are rather understudied. *Rickia wasmannii* Cavara is a common ant-associated Laboulbeniales species that has been reported in 17 countries of Europe, and frequently infects *Myrmica scabrinodis* Nylander, 1846 (Hymenoptera: Formicidae), a common ant species host, in high density. These make *M. scabrinodis* and *R. wasmannii* appropriate model organisms for studies on fungal host-ectosymbiont interactions. Aggressiveness and boldness of infected and uninfected *M. scabrinodis* workers from northern and eastern Hungary were studied in two laboratory-established behavioural experiments. Infected workers were significantly less aggressive and less bold (i.e. less likely to leave nest shelters) than the uninfected ones. These results suggest that *R. wasmannii* has considerable effects on the behaviour of *M. scabrinodis*. Our study brings an evidence that infection of ants with Laboulbeniales might negatively affect the workers' behaviour. In special, the competitive abilities might be affected most by these fungi, since remaining inside and behaving submissively is not effective behaviour in the case of significant competition for resources among colonies.

Keywords

behaviour, ectoparasite, Laboulbeniomycetes, fungi, infection spreading

Introduction

The ascomycete order Laboulbeniales contains 141 genera and over than 2,100 species of obligatory ectosymbionts (Santamaria et al. 2017). The hosts of these fungi mainly belong to the groups Coleoptera (ca. 80%) and Diptera (ca. 10%) (Weir and Hammond 1997). Most Laboulbeniales fungi exhibit extreme host specificity, with a single host species (stenotopic) or several species in the same genus (eurytopic) (Haelewaters 2012 and references therein), but unrelated insects can also host a single Laboulbeniales species if they share the same microhabitat (De Kesel and Haelewaters 2014, Pfliegler et al. 2016). While such ectosymbiotic associations can have both positive (Konrad et al. 2015) and negative (Benjamin 1971, Gemeno et al. 2004, Nalepa and Weir 2007, Strandberg and Tucker 1974) effects on their hosts, they are sometimes considered to be neutral factors (García et al. 2010, Lapeva-Gjonova and Santamaria 2011, Whisler 1968).

To date, four ant-associated species of Laboulbeniales have been recorded in Europe: *Laboulbenia formicarum* Thaxt. is known in France, Portugal and Spain, parasitizing *Lasius grandis* Forel, 1909, *L. neglectus* van Loon, Boomsma & Andrasfalvy 1990 and *L. niger* (Linnaeus, 1758) (Espadaler and Santamaria 2012, Gómez et al. 2016). *Laboulbenia camponoti* S.W.T. Batra is known in Bulgaria, Spain, Austria, Romania and Italy, on *C. universitatis* Forel, 1890; *C. pilicornis* (Roger, 1859); *C. sylvaticus* (Olivier, 1792) and *C. aethiops* (Latreille, 1798) (Báthori et al. 2014, Espadaler and Santamaria 2012; Gómez et al. 2016). *Rickia lenoirii* Santam. has been reported in Greece, France, Hungary and Romania on *Messor wasmanni* Krausse, 1910 and *M. structor* (Latreille, 1798) (Báthori et al. 2015b, Santamaria and Espadaler 2015). *Rickia wasmannii* Cavara (Fig. 1) is the most widely distributed ant-associated Laboulbeniales in Europe (Santamaria and Espadaler 2015). This species has been reported from England, France, Luxemburg, Germany, the Netherlands, Switzerland, Poland, Italy, Austria, Slovenia, the Czech Republic, Slovakia, Hungary, Romania, Spain, Belgium and Bulgaria, and parasitizes nine *Myrmica* Latreille, 1804 species: *M. gallienii* Bondroit, 1920; *M. rubra* (Linnaeus, 1758); *M. ruginodis* Nylander, 1846; *M. sabuleti* Meinert, 1861; *M. scabrinodis* Nylander, 1846; *M. slovaca* Sadil, 1952; *M. specioides* Bondroit, 1918; *M. spinosior* Santschi, 1931 and *M. vandeli* Bondroit, 1920 (De Kesel et al. 2016, Haelewaters et al. 2015a, 2015b).

Ant species within the genus *Myrmica* can be found in several different kinds of habitats across European temperate zones, including meadows, steppes, woodlands, forests, and mountainous regions. Colonies of these ants can be either monogynous or polygynous and contain up to a few thousand workers (Radchenko and Elmes 2010, Czechowski et al. 2012). *Myrmica* ants can be hosts to many parasitic organisms from several taxonomic groups including ecto- and endoparasitic fungi and other microbial pathogens, such as: *R. wasmannii*; *Beauveria bassiana* (Bals.-Criv.) Vuill.; *Isaria fumosorosea* Wize; *Metarhizium anisopliae* (Metschn.) Sorokin; *Hirsutella stibelliformis* var. *myrmicarum* H.C. Evans & Groden; *H. subramanianii* var. *myrmicarum* H.C. Evans & J.F. Bischof; *Paraisaria myrmicarum* H.C. Evans, Groden & J.F. Bischof; *Ophiocordyceps myrmicarum* D.R. Simmons & Groden (Radchenko and Elmes 2010, Simmons et al. 2015, Witek et al. 2014). In particular, *M. scabrinodis* is common in Europe

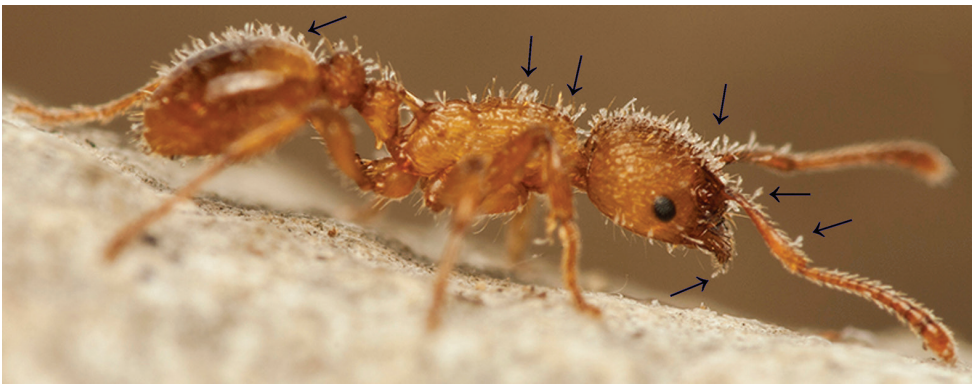

Figure 1. *Myrmica scabrinodis* worker with *Rickia wasmannii* thalli (arrows indicate some thalli).

(Czechowski et al. 2012, Radchenko and Elmes 2010, Seifert 2007), frequently parasitized by *R. wasmannii* (Espadaler and Santamaria 2012) and other (social) parasites (Tartally 2008, Witek et al. 2014). *Myrmica scabrinodis* is a mesothermo- and mesohygrophilic species of different kinds of humid habitats, which is tolerant of soil moisture but avoids expressly xerothermal habitats. The abundance of both *R. wasmannii* and *M. scabrinodis* make them appropriate species for the study of host-Laboulbeniales interactions, as corroborated by a number of recent studies (Báthori et al. 2015a, Csata et al. 2014, Haelewaters et al. 2015a, 2015b, Markó et al. 2016, Witek et al. 2014).

Previous work has shown that different ant-fungal interactions can influence the behaviour of hosts in several ways (e.g., increased grooming and nest cleaning, secretion of antibiotics, pathogen avoidance, dispersal of infected individuals, and the relocation of an entire colony) (e.g., Csata et al. 2014, Roy et al. 2006, Oi and Pereira 1993). Laboulbeniales fungi are often present on the ant body with high thallus densities and the results of recent studies suggest that they could have both positive and negative effects on their hosts and are able to influence their behaviour (Báthori et al. 2015a, Csata et al. 2014, Konrad et al. 2015, Pech and Heneberg 2015). For example, Csata et al. (2014) showed that individuals of *M. scabrinodis* infected by *R. wasmanni* had significantly reduced lifespans under laboratory conditions; heavily infected individuals died significantly faster from starvation and spent more time consuming water than their more lightly parasitized counterparts. Starvation survival of *L. neglectus* workers infected by *L. formicarum* was also significantly decreased (Konrad et al. 2015), while host survival due to *Metarhizium* exposure increased. Ant workers with high thallus densities exhibited significantly longer periods of self-grooming as well as elevated expression of immune genes (Konrad et al. 2015).

To date, it has not been studied whether the boldness and aggressive behaviour differ between infected and uninfected *M. scabrinodis* workers. The behavioural characteristics of different ant workers could be relevant, for example, in terms of competition between infected and uninfected colonies. In this study, we evaluate the boldness and aggressive behaviour of uninfected *M. scabrinodis* workers versus their heavily infected counterparts.

Methods

Ant colony collection and laboratory conditions

We collected *Myrmica scabrinodis* colonies from two different regions in Hungary, including 12 from northern Hungary which comprised six which were infected from a habitat close to Rakaca (i.e., 48°27'N, 20°47'E, 170 m above sea level, a.s.l.) and six that were uninfected from an area close to Aggtelek (i.e., 48°26'N, 20°30'E, 340 m a.s.l.). We collected 12 further colonies from eastern Hungary, including six that were infected from close to Újléta (i.e., 47°26'N, 21°51'E, 120 m a.s.l.) and six that were uninfected from close to Csíkgát (47°25'N, 21°48'E, 110 m a.s.l.). We did not record any sites as part of this study that comprised both infected and uninfected colonies, so a standardisation of experiments per locality by choosing both infected and uninfected colonies from the same sites was unfortunately not possible. However, note that the two paired-sites were in a similar part of Hungary and from similar habitats and elevations, for sake to minimize the environmental effects on the populations. Thus, our total dataset includes 24 colonies, each of which contained fertilized queens and hundreds of workers, larvae, and pupae. To further reduce the effect of different sites, we kept all the ants used for this study for minimum two weeks in artificial lab nests under the same conditions: at 20 ± 1°C and provided sufficient food resources (i.e., fed with cockroaches twice a week and with a 33% honey water solution *ad libitum*). Plastic boxes treated with Fluon® on their inner walls to prevent ants from escaping were used as formicaria (i.e., length: 16.5 cm; width: 11.5 cm; height: 6 cm); inside these boxes, we created chambers (i.e., length: 5.5 cm; width: 4.5 cm; height: 1 cm) with plaster floors, covered with glass plates. In advance of each experiment, colonies were stored in the laboratory for a minimum of one month for acclimatisation (following Báthori et al. 2015a).

Boldness test

Boldness was tested using individual workers. We assessed their boldness by measuring how long it took for them to leave shelter (based on Gyuris et al. 2011). Prior to the test, 18 melanised workers were randomly selected from each colony (total n = 432). These mini test-colonies were fed a Bhatkar diet (Bhatkar and Whitcomb 1970). After 12 hours, single individuals were placed into different shelters in random order, inside new sterilized thin and plugged black plastic tubes (length: 60 mm; diameter: 5 mm) for an acclimatisation period of one minute (Gyuris et al. 2011, Spicer Rice and Silverman 2013) (Fig. 2a). After the removal of the plug, we measured the time that passed before individual ants emerged from the tubes, allowing a maximum waiting time of three minutes (see Báthori et al. 2015a).

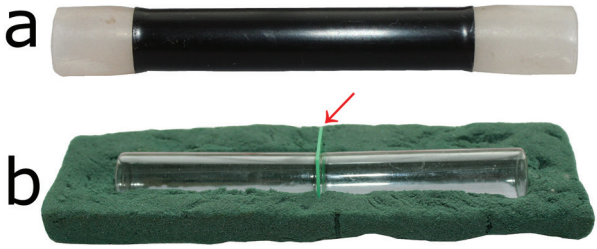

Figure 2. Tubes used for **a** boldness and **b** aggression tests. Arrow indicates the flap which was removed at the beginning of the test.

Aggression test

In this experiment, 120 worker pairs, including one infected and one uninfected melanised worker, were randomly selected from both geographic regions. Worker pairs from the two different geographical regions, northern and eastern Hungary were tested separately, and two facing glass tubes (length: 53 mm; diameter: 15 mm), separated by a thin plastic flap, formed the experimental arena (Fig. 2b). After an acclimatisation period of 1 minute (Spicer Rice and Silverman 2013), the flap was removed so that the infected and uninfected workers could meet. The number of different behaviour patterns was registered for a period of 3 minutes (like in Báthori et al. 2015a). The following categories were used: initiation of aggressive behaviour, antennation, mandibular threats, biting, stinging, autogrooming and allogrooming (see Maák et al. 2014 and references therein). At the end of the experiment, the ants were preserved in a solution of 67.5% ethanol, and the number of fungal thalli was counted for statistical analyses (see below) under a Leica MZ12.5 stereomicroscope at magnifications of 10-160x.

Statistical analyses

Statistical analyses were performed using the R statistical software (ver. 3.0.2, R Core Team 2013). Data was mainly analysed using generalized linear mixed-effects models (GLMM) of the R-package "lme4" (Bates et al. 2014), because these types of models offer a range of ways to handle non-Gaussian error distributions and random effects. In GLMMs of significant correlations we acquired conditional R^2 of the model using the R-package "MuMIn" (Barton 2013).

To assess whether there was significant difference between infected and uninfected workers in terms of their probability to leave shelter, we applied a binomial GLMM, specifying leaving shelter as binary response (1 meaning the individual left shelter), and infection as binary predictor factor (1 meaning the individual was infected).We also tested if infected and uninfected workers showed significant differences in their latency before leaving shelter by fitting a mixed-effects Cox regression model in the R-package

'coxme' (Therneau 2016). In this model, time latency before leaving the shelter was defined as follow up time, while the event of leaving was specified as the status indicator event, and infection was a binary predictor factor variable. To test whether the number of thalli on infected workers affected the time latency before leaving shelter, we fitted a log-linked Gamma GLMM, because this model is able to handle Gamma-distributed time data. When fitting this Gamma GLMM, we excluded observations from uninfected workers and defined the number of thalli as predictor, while using time latency before leaving shelter as response variable. In all the models described above, the time-block of measurements and the habitat of origin were specified as random factors.

In order to measure aggressiveness, we calculated an index based on Martin et al. (2009), dividing the number of observed aggressive behaviours (mandible threat, biting, stinging) by the total number of interacting behaviours (mandible threat, biting, stinging, antennae-interaction, and allogrooming). This index represent the proportion of aggressive behaviours in the overall observed and recorded interactive behaviours. When testing whether infection has a significant effect on the proportion of aggressive behaviours, we fitted a binomial GLMM, with infection as the predictor factor, and calculated aggression index as dependent variable. To see if infected or uninfected workers were more likely to initiate aggressive interactions, we used a different binomial GLMM that incorporated infection as a predictor, and initiation (binary variable, 1 meaning that the given ant was the first to perform aggressive behaviour in the given trial) as a dependent variable. In the two models described above, the ID of tested pairs and habitat of origin were specified as random factors. Furthermore, we tested whether, the number of thalli had any effect on the proportion of aggressive behaviour by fitting a binomial GLMM (excluding all uninfected ants) with aggression index as the dependent and the number of thalli as the predictor variable. In this model, habitat of origin was also specified as a random factor.

Results

Boldness

Infected ants did not differ from uninfected ones in their probability of leaving shelter (binomial GLMM: $z = -0.99$; $p = 0.318$). However, infected workers were significantly slower to leave shelter, i.e. showed higher latencies before leaving (mixed-effects Cox-model: $z = -2.13$; $p = 0.033$) (Fig. 3). The number of thalli on infected workers did not affect significantly the time latency before leaving shelter (log-linked Gamma GLMM: $z = -0.33$; $p = 0.744$).

Aggression

Infection alone does not exert a significant effect on the proportion of aggressive behaviours (binomial GLMM: $z = -0.41$; $p = 0.68$). Infected workers were, however,

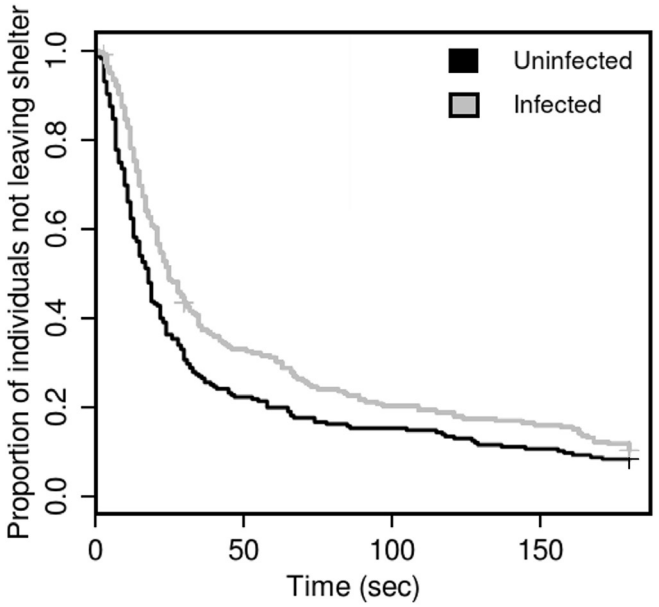

Figure 3. Time latency until leaving shelter in case of uninfected (N=215) and infected (N=215) workers.

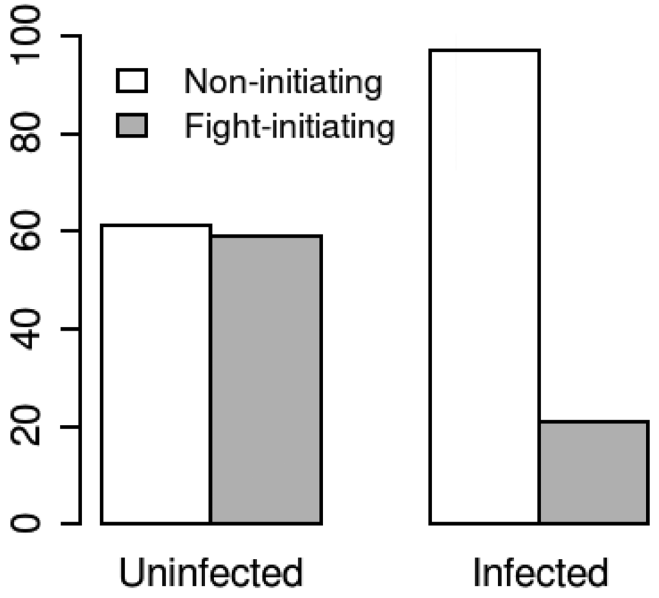

Figure 4. Probability if initiating aggressive behaviour in cases of uninfected (N=119) and infected (N=119) workers.

significantly less likely to initiate aggressive behaviour than uninfected ants (binomial GLMM: z = -2.91; p = 0.004; conditional R^2 = 0.23) (Fig. 4). Interestingly, the number of thalli on infected workers nevertheless seems to exert a mild, but significantly positive effect on the proportion of aggressive behaviours (binomial GLMM: z = 2.85; p = 0.005; conditional R^2 = 0.50).

Discussion

On the basis of our experimental results, *R. wasmannii* seems to have a considerable effect on the behaviour of *M. scabrinodis* workers with regards to boldness and interactions with uninfected workers. Since infection by the fungus appears to be permanent (Haelewaters et al. 2015c, AT and FB pers. observ. on long-term reared laboratorial colonies), this could lead to persistent differences in the ants' behaviour in a given colony (De Kesel et al. 2016). It is worth noting that when a colony is infected, the vast majority of its workers are also infected (Markó et al. 2016; AT and FB, pers. observ.), and the infection therefore could have effects not only on the individual but also on the colonial level. Thus, infection by *R. wasmannii* possibly has long-term effects on the performance of infected individuals and colonies in natural habitats. It should be noted here that we had the possibility to work with populations with either infected or uninfected colonies from slightly different sites (see Methods for details). Thus, it would be important to follow similar experiments on infected and uninfected colonies collected from the same site, if such sites would be recorded in the future to confirm that our results are not affected by habitat-driven differences of maternal colonies, although it is here unlikely. It should be also emphasized that so far all results about negative and positive interactions were reached in the course of laboratory experiments, so additional experiments are needed to conduct with the ant hosts in nature.

We propose the following hypotheses regarding the increased latencies in time of leaving shelter in the case of infected workers: (1) they may have been in poorer condition and physically weak or impaired, e.g. the chances of survival of infected ants were lower under starvation (Konrad et al. 2015) and lab conditions (Csata et al. 2014); and/or (2) the fungus induced the host to remain in the shelter tube for a longer period of time. If the latter were true, this would mean that *R. wasmannii* would induce behavioural changes. It should be noted that Csata et al. (2017) did not found any differences in the locomotion of infected worker specimens compared with the uninfected ones. Thus, the infected ants did not left the tube later because of locomotion but rather because of other behavioural changes (i.e. boldness). The mechanisms causing infected ants to stay longer in the shelter could be of importance, and should be the subject of future study.

On the other hand, *R. wasmannii*, as an ectosymbiotic fungus, could be expected to be capable of spreading not only within but also between ant colonies. If increased worker activity (outside of the nest) would increase the spreading success of the fungus, one may expect infected ants to exhibit a higher degree of out-of-nest activity (Bos et al. 2012, sensu Hughes et al. 2011). Where a host forages more outside and fights, this would have a positive effect on the spread of the fungus compared to the behaviour of a submissive host who stays inside the nest. Furthermore, local competitive disadvantages that influence infected colonies (see: Báthori et al. 2015a, Csata et al. 2014) in a given habitat might be reduced by spreading the infection to local rival colonies. However, our results show that the infected *M. scabrinodis* workers do not behave in these ways. Thus, the intercolonial spread of *R. wasmannii* caused by individual foragers might be rare and occasional. It would need further research to check whether the fungus rather spreads by queens or by

whole colony buddings, a phenomenon that is well-known in *Myrmica* ants (Radchenko and Elmes 2010). Spreading mainly by colony budding would suggest an extreme host specialisation of the fungus on the host ant colonies. Such an extreme host colony specialization is recorded in the case of at least one ant-parasitic species [the social parasite *Microdon mutabilis* (Linnaeus, 1758) hoverfly (Diptera: Syrphidae); see Schönrogge et al. 2006], but since currently no information is available on the genetic relationships among different *R. wasmannii* populations, it is impossible to determine whether or not fungus lineages are similarly adapted genetically to the different host ant colonies (and thus infecting genetically distant host colonies would not be effective). Furthermore, we cannot exclude the possibility that the spreading of the fungus requires the specific microhabitat provided by the ant nest [compare to the ecological specificity of *R. wasmannii*, (Pfliegler et al. 2016)], e.g. because the ascospores of *Rickia* ejected from mature thalli are extremely vulnerable to desiccation outside the ant nest microhabitat. Such hypothetical constraints on the spreading of the infection could actually act in favour of the observed behavioural effects on the host, or at least may account for the lack of negative selection regarding less active workers. In this sense, the poorer general condition of the infected host that results in less active behaviour would mean an advantage for the fungus.

For a long time, there was no knowledge of the effects of ant-associated Laboulbeniales fungi on the behaviour of their hosts, but recent research (Báthori et al. 2015a, Csata et al. 2014, Konrad et al. 2015) has begun to fill this gap. These studies revealed behavioural changes in the ant hosts, such as increased water consumption (Báthori et al. 2015a) and intensified grooming (Csata et al. 2014). Based on our current results, infection might negatively affect also the workers' behaviour linked to competitive abilities, since remaining inside and behaving submissively is not effective when there is significant competition for resources.

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